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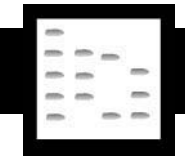
DYES



High-Speed Countercurrent Chromatography

A. Weisz, Food and Drug Administration, Washington DC, USA
Y. Ito, National Institutes of Health, Bethesda, MD, USA

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Countercurrent chromatography is one of the liquid–liquid partition chromatographic techniques that do not use a solid support. High-speed countercurrent chromatography (HSCCC) uses centrifugal force to retain one of the liquid phases in an lto multilayered coil column while the second liquid phase is pumped through the column. The principles of this technique have been discussed by Ito.

HSCCC in its two forms, conventional and pH-zone-refining CCC, is relatively new among the preparative techniques used for the separation of dyes. Conventional HSCCC has been applied to this purpose since the mid-1980s when Fales *et al*. separated various components present in a sample of the triphenylmethane biological stain, Methyl Violet 2B, and when Freeman and co-workers purified azo textile and ink dyes (i.e. acid, direct and disperse azodyes). This technique was subsequently used for the separation and purification of components from other colours, such as D&C Red No. 28 (Phloxine B, CI 45410), Sulforhodamine B (CI 45100) and Gardenia Yellow. It was also effectively implemented as a complement to preparative high performance liquid chromatography (HPLC) for the separation of a complex synthetic mixture of brominated tetrachlorofluorescein dyes. Conventional HSCCC was applied to the separation of quantities of dyes of up to several hundred milligrams when the common 1.6 mm i.d./325 mL volume column was used. By contrast, the more recently developed (1993) pH-zone-refining CCC was applied from the outset to the separation of multi-gram quantities of dye mixtures such as xanthene and fluoran dyes used as colour additives in food, drugs or cosmetics and as biological stains.

Using a modified procedure, the applications of this technique have been extended to the separation of gram quantities of the highly polar mono-, di- and trisulfonated components of D&C Yellow No. 10 and Yellow No. 203 (both Quinoline Yellow, CI 47005) and of other sulfonated dyes such as FD&C Yellow No. 6 (Sunset Yellow, CI 15985) and D&C Green No. 8 (Pyranine Concentrated, CI 59040). A general approach to the separation of dyes by HSCCC is presented in **Figure 1**.

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Instrumentation

For the separation of dyes, both by conventional and pH-zone-refining CCC, commercially available high speed CCC centrifuges are used. Such instruments are described in the HSCCC entry in this volume. The separations described below were performed with HSCCC systems from PC, Potomac, MD, USA and Pharma-Tech Research, Baltimore, MD, USA. A schematic diagram of a HSCCC system used for separation of dyes is shown in **Figure 2**.

Conventional HSCCC

Conventional HSCCC may be applied only to the separation of relatively small amounts (up to several hundred milligrams when the common 325 mL volume column is used) of dye mixtures. In contrast to pH-zone-refining CCC, conventional HSCCC also permits separation of nonionic components. Analytical size separations (less than 10–20 mg of an ionic or nonionic component of interest) should be performed by conventional HSCCC.

Selection of the Solvent System

The solvent system used for a conventional HSCCC separation is selected according to the hydrophobicity

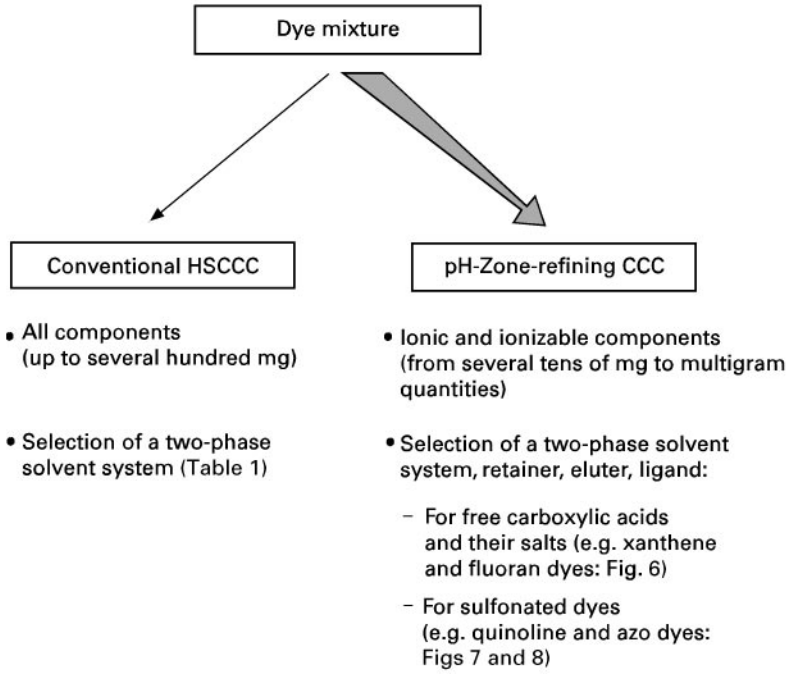

Figure 1 General approach to the preparative separation of dyes by HSCCC.

of the targeted components so that the respective partition coefficients (K) of these components are close to 1. **Table 1** summarizes the solvent systems used for specific dye separations. Also listed in Table 1 are the general requirements for the selection of a solvent system. The procedures for a systematic search for a suitable solvent system have been discussed previously.

Separation Procedure

The separation is initiated by filling the column with the stationary phase (usually the upper organic phase of the biphasic solvent system) using the liquid chromatography pump (Figure 2). The sample to be separated, dissolved in a minimum volume of the solvent system (equal volumes of each phase), is then loaded into the column through the sample injection valve (which facilitates the elimination of air bubbles) by syringe or with pressurized nitrogen (60 psi). The mobile phase is then pumped into the column while the column is rotated (usually at 3 mL min^{-1} and 800 rpm). The effluent is passed through a UV-visible detector, and fractions of 3 or 6 mL per test tube are collected with the aid of a fraction collector. The fractions obtained from the HSCCC separation are then further analysed (e.g. by HPLC).

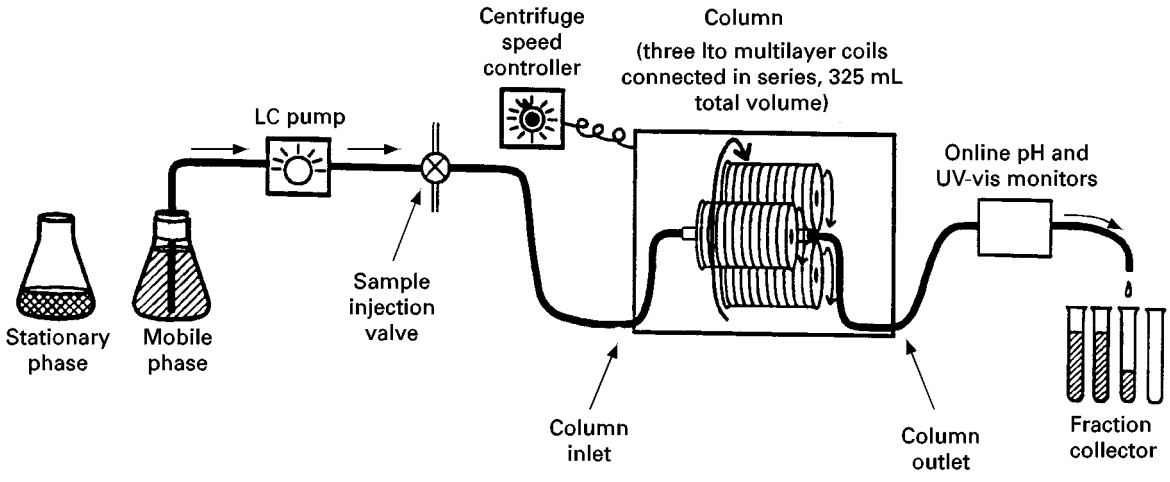

Figure 2 Schematic presentation of a HSCCC system.

Table 1 Selection of a two-phase solvent system for conventional HSCCC of dyes

Reported solvent systems
Methyl Violet 2B ($CHCl_3$–AcOH–0.1 mol L^{-1} HCl (2 : 2 : 1))
Sulforhodamine B (*n*-BuOH–0.01 mol L^{-1} aq trifluoroacetic acid (1 : 1))
Gardenia Yellow (EtOAc–*n*-BuOH–H_2O (2 : 3 : 5))
Azo-dyes (acid, direct and disperse azo-dyes)
Phloxine B (CI 45410) (EtOAc–*n*-BuOH–0.01 mol L^{-1} aq NH_4OAc (1 : 1 : 2))
Brominated tetrachlorofluoresceins (EtOAc–*n*-BuOH–0.01 mol L^{-1} aq NH_4OAc (1 : 1 : 2))
New solvent systems: general requirements
Settling time shorter than 30 s
Partition coefficient (K) close to 1 and should be different for each component of the dye mixture
Solvent system should provide nearly equal volumes of upper and lower phases

Applications

Table 1 summarizes the applications of conventional HSCCC to the separation of components from synthetic and natural dye mixtures. **Figures 3** and **4** show two examples in which this technique was successfully applied to the separation of dye mixtures.

Oka *et al*. employed conventional HSCCC to separate the main components from the gardenia fruit extract (*Gardenia jasminoides* Ellis) used extensively in Japan as a food colour additive under the name Gardenia Yellow (Figure 3). The solvent system used for the separation, ethyl acetate–*n*-butanol–water (2 : 3 : 5), was chosen based on the partition coefficient (K) of each of the 14 components, as determined by HPLC. Figure 3A shows the HPLC analysis of the original mixture. The chromatogram obtained for the separation of the three main components (geniposide, *trans*-crocin and 13-*cis*-crocin) from a 25 mg portion of Gardenia Yellow by conventional HSCCC, using the upper organic layer as the stationary phase, is shown in Figure 3B.

Figure 4 shows the chromatogram obtained by Fales *et al*. for the HSCCC separation of the components of a 6 mg portion of the triphenylmethane dye Methyl Violet 2B, composed of a mixture of *N*-methylated forms of pararosaniline. The solvent system used in this case, $CHCl_3$–acetic acid–0.1 mol L^{-1} HCl (2 : 2 : 1), succeeded in separating the various methylated homologues and several contaminants. For this separation, the aqueous layer was used as the stationary phase. The proposed structures of the separated components and of one of the isolated contaminants (mol.wt 491; Figure 4) are based on their ^{252}Cf plasma desorption mass spectra.

pH-Zone-Refining CCC

pH-zone-refining CCC can only be applied to the separation of ionic or ionizable components. The principles of this technique are discussed in this volume. Ideally, the sample components should be stable over a wide pH range, from 1 to 10 for dyes containing a carboxylic acid group and from 0.5 to 13.5 for sulfonated dyes. The quantity of each targeted component in the sample mixture must be at least 0.1 mmol and preferably over 1 mmol. Depending on the solubility of the sample mixture in the solvent system, multi-gram quantities of dyes can be separated in one experiment using a typical preparative column of approximately 325 mL capacity. A general approach to the separation of dyes by pH-zone-refining CCC is presented in **Figure 5**. This method is applied to the separation of dyes containing carboxylic acid groups (e.g. xanthene dyes or their lactone analogues, fluoran dyes) by using an organic acid (e.g. trifluoroacetic acid, TFA) as a retainer in the organic stationary phase and a base (e.g. ammonia) as an eluter in the aqueous mobile phase. Compounds containing one or more amine groups (e.g. Methyl Violet 2B) may also be separated by pH-zone-refining CCC by using an organic base as a retainer (e.g. triethylamine) and an inorganic acid as an eluter (e.g. HCl), as described elsewhere in the present volume.

The more polar sulfonated dyes can be separated by affinity-ligand pH-zone-refining CCC (right column in Figure 5), in which case a ligand is added to enhance the partitioning of the dye components into the organic stationary phase.

Selection of the Solvent System

The process of selecting a solvent system for pH-zone-refining CCC separations is very different from that conducted when selecting a solvent system for conventional HSCCC separations. For most pH-zone-refining CCC separations, a suitable solvent system can be found by testing various volume ratios of ether (diethyl or methyl-*tert*-butyl, MTBE)–acetonitrile (CH_3CN)–water. To the chosen system, an or-

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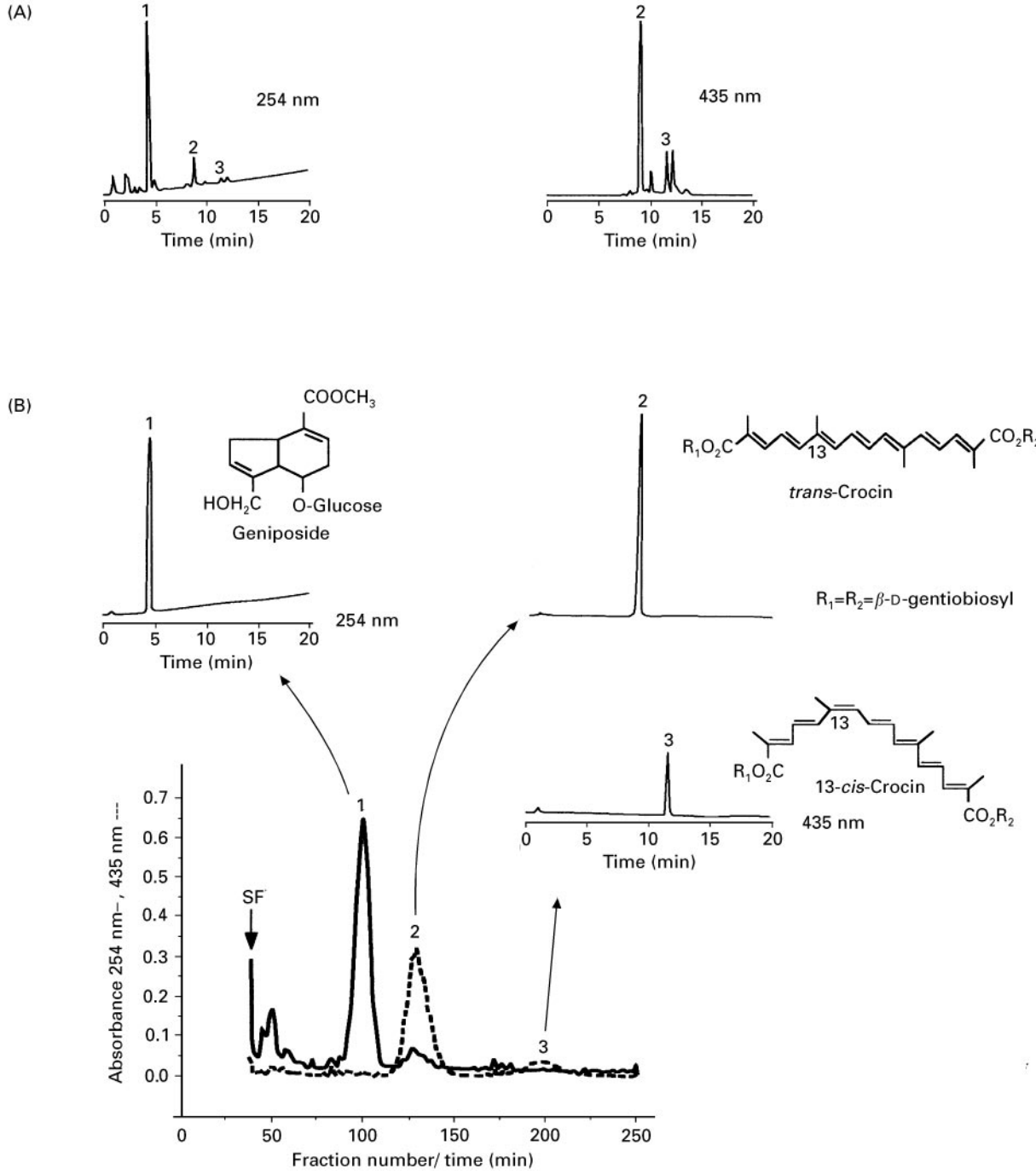

Figure 3 Separation by conventional HSCCC of components from a sample of the Japanese food colour additive Gardenia Yellow. (A) High performance liquid chromatograms at 254 nm and 435 nm of the original sample. (B) Conventional HSCCC for the separation of a 25 mg portion of the sample and the HPLC chromatograms of the separated components. Experimental conditions: solvent system: ethyl acetate–*n*-butanol–water (2 : 3 : 5 by volume). The aqueous phase was used as mobile phase. Sample: 25 mg Gardenia Yellow dissolved in 2 mL solvent (1 mL of each phase). Flow rate: 2 mL min^{-1} in the head-to-tail elution mode. Detection: 254 (continuous line) and 435 nm (dashed line). Speed of revolution: 800 rpm. Stationary-phase retention: 65.8%. SF, Solvent front. (Oka *et al.*, 1995 with modifications.)

ganic acid (TFA) is added to the organic layer as a retainer and an inorganic base such as ammonia is added to the aqueous layer as an eluter if dyes containing carboxylic acid groups are to be separated. For the separation of dyes containing amino groups, an organic base (such as triethylamine) is added to the organic stationary phase as a retainer and an inorganic acid (HCl) is added to the aqueous

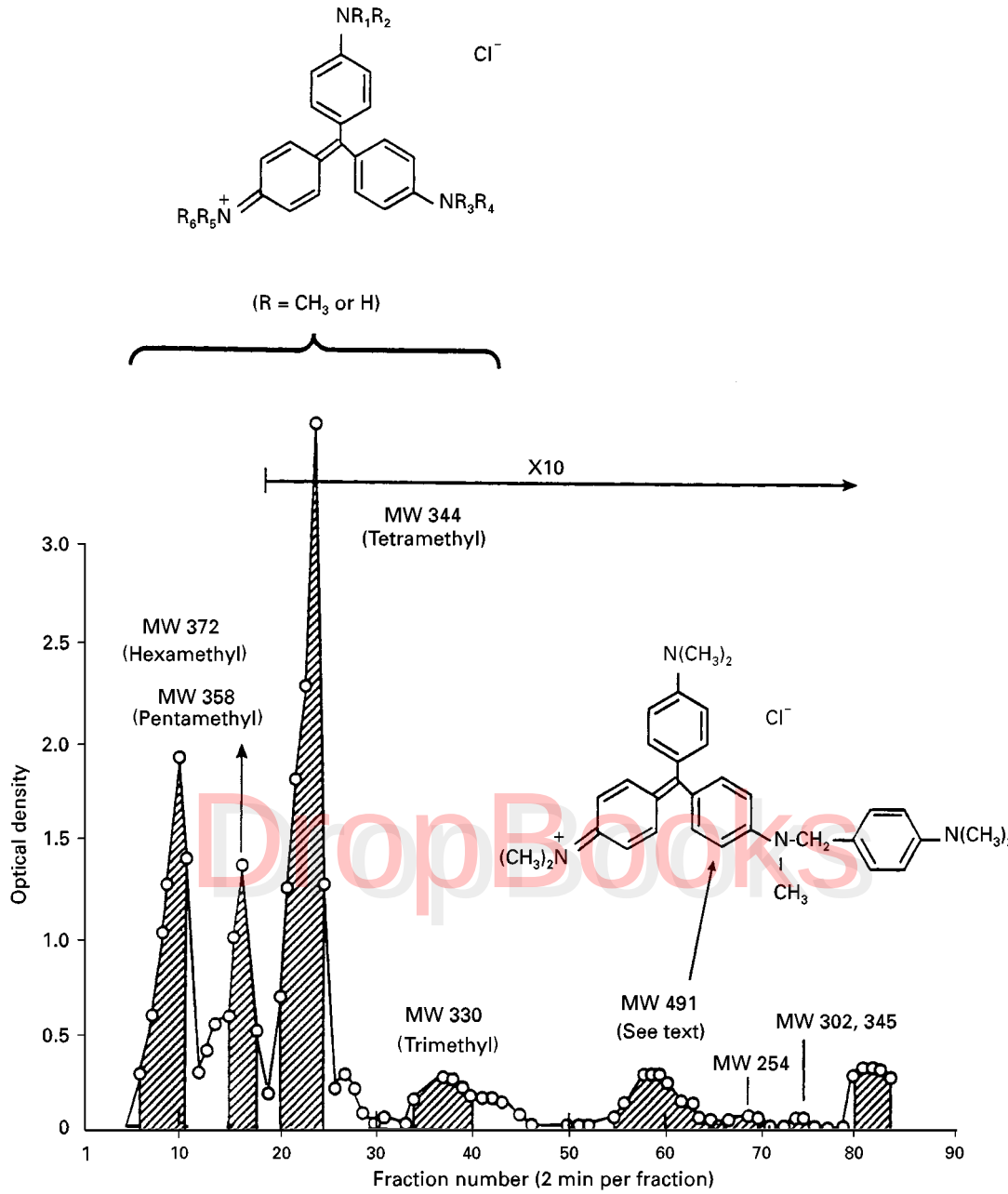

Figure 4 Separation by conventional HSCCC of components from a sample of Methyl Violet 2B. Experimental conditions: Solvent system: chloroform–acetic acid–0.1 mol L^{-1} HCl (2 : 2 : 1 by volume). The organic phase was used as mobile phase. Sample: 6 mg Methyl Violet 2B dissolved in 5 mL organic phase. Flow rate: 4 mL min^{-1} in the head-to-tail elution mode. Speed of revolution: 800 rpm. (Fales *et al.*, 1985 with modifications.)

mobile phase as an eluter. For affinity-ligand pH-zone-refining CCC separations (for sulfonated dyes), a ligand is added to the stationary phase to retain the sulfonated dyes in the column by enhancing their partitioning into the organic stationary phase.

Standard pH-zone-refining CCC The following steps are recommended for the selection of an appropriate two-phase solvent system for the separation by standard pH-zone-refining CCC of a dye containing a carboxylic acid group (summarized in **Figure 6**):

1. Prepare a two-phase solvent system by thoroughly equilibrating water and either MTBE or diethyl ether in a separatory funnel at room temperature.

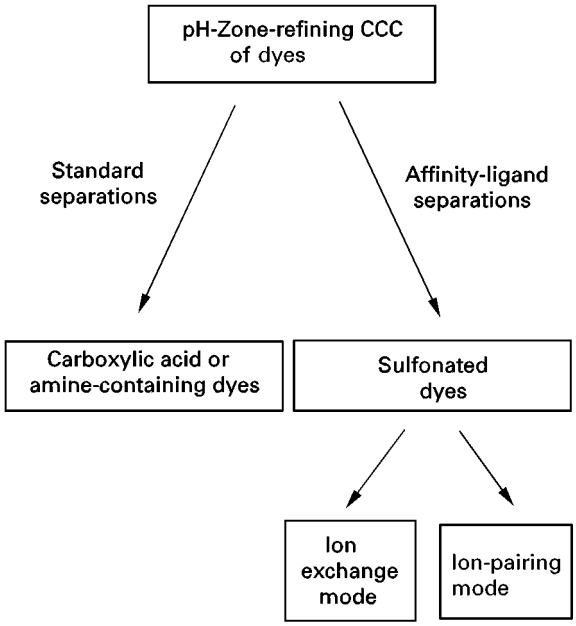

Figure 5 General approach to the separation of dyes by pH-zone-refining CCC. (Reproduced from Fales *et al.*, 1985 with permission from the American Chemical Society.)

2. Deliver a 2 mL aliquot of the upper (U) and of the lower (L) phase into a test tube. Add a very small amount of dye and agitate to equilibrate the contents.
3. Add a small amount of aqueous ammonia (≈28%; eluter) to the mixture (to give a base concentration of approximately 12 mmol L^{-1}, pH 10) and equilibrate the mixture. If almost all the colour visibly partitions into the lower aqueous phase, then the partition coefficient $K_{base}(U/L) \ll 1$. If visual assessment is unclear, K_{base} may be determined by spectrophotometry. Dilute an aliquot from each phase with solvent (e.g. methanol) and measure the absorbance at an appropriate wavelength using a spectrophotometer. Obtain the partition coefficient, $K_{base}(U/L)$, by dividing the absorbance of the dye in the upper phase by that in the lower phase.
4. If $K_{base} \geq 0.5$, the above test should be repeated with a less polar (more hydrophobic) solvent system such as *n*-hexane–ethyl acetate–methanol–water (1 : 1 : 1 : 1).
5. If $K_{base} \ll 1$, add retainer acid TFA (approximately 20 mmol L^{-1}) to the mixture to bring the pH to approximately 2, and re-equilibrate the mixture by agitation. Using procedure 3, obtain K_{acid}. If $K_{acid} \gg 1$, the solvent system can be effectively used to separate the sample components.
6. If $K_{acid} \leq 2$, the above tests should be repeated with a more polar (more hydrophilic) solvent system such as *n*-butanol–water.

For amine-containing dyes, substitute HCl for ammonia in step 3 above to obtain $K_{acid} \ll 1$ and substitute triethylamine for TFA in step 5 above to obtain $K_{base} \gg 1$.

Affinity-ligand separations Sulfonated dyes are much more polar (hydrophilic) compounds than the carboxylic acid dyes. They tend to distribute predominantly in the aqueous phase of a conventional two-phase solvent system, even if the aqueous phase is highly acidic. For the separation of sulfonated dyes by pH-zone-refining CCC, the addition of a hydrophobic ligand is necessary in order to retain the dyes in the organic stationary phase. Two kinds of ligands were used successfully for the separation of sulfonated dyes: ion exchange reagents (e.g. dodecylamine, tridodecylamine), which are always retained in the organic stationary phase, and

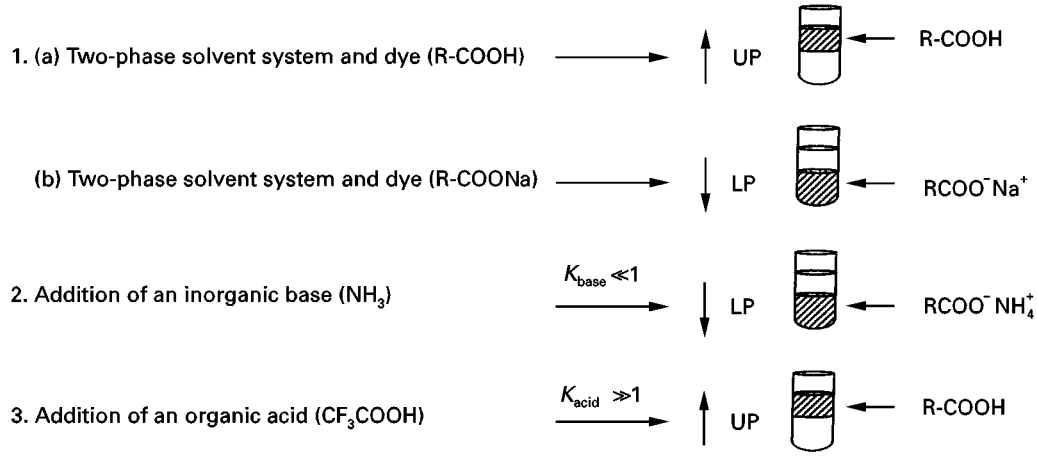

Figure 6 Conditions required for the separation of dyes containing carboxylic acid groups by pH-zone-refining CCC. For conditions required for the separation of dyes containing amino groups, see text.

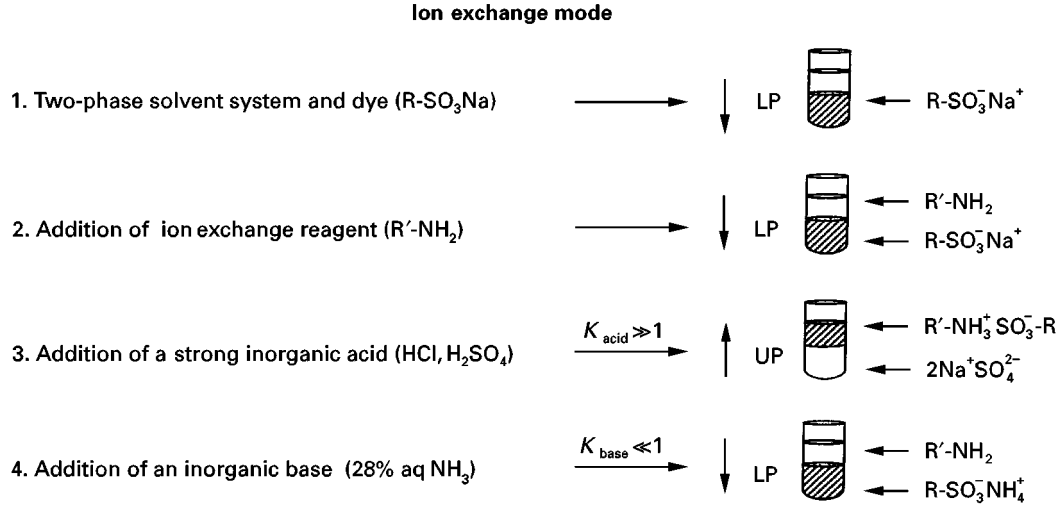

Figure 7 Conditions required for the separation of sulfonated dyes by affinity-ligand pH-zone-refining CCC in the ion exchange mode.

ion-pairing reagents (e.g. tetrabutylammonium hydroxide), which partition into either phase. These ligands are dissolved in the organic phase (stationary phase and/or sample solution) at a concentration determined in preliminary experiments.

Ion exchange mode separations (**Figure 7**) The following steps are recommended for the selection of an appropriate two-phase solvent system for the separation of sulfonated dyes by affinity-ligand pH-zone-refining CCC in the ion exchange mode:

1. Prepare a two-phase solvent system composed of either MTBE–CH_3CN–water at a volume ratio of 2:2:3 (used for the separation of the main component from FD&C Yellow No. 6, Sunset Yellow, CI 15985) or iso-amyl alcohol (iAA)–MTBE-CH_3CN–water at volume ratios from 3 : 1 : 1 : 5 to 3 : 5 : 1 : 7 (used for the separation of mono-, di- and trisulfonated components of Quinoline Yellow, CI 47005).
2. Deliver a 2 mL aliquot of the upper (U) and of the lower (L) phase into a test tube. Add a small amount of dye and agitate to equilibrate the contents.
3. Add a small amount of ion exchange reagent (e.g. dodecylamine, tridodecylamine at 5–20% concentration) and equilibrate the mixture by agitation.
4. Add a small amount of a strong inorganic acid (e.g. HCl, H_2SO_4 at 3–4%, pH 0.8). Measure the K_{acid} as above.
5. If $K_{acid} \leq 5$, add more acid and retest.
6. If $K_{acid} \gg 1$, add eluter NH_3 (28% aq NH_3) to the mixture to give a base concentration of approximately 110 mmol L^{-1} and a pH of 11. Re-equilibrate the contents. Measure the K_{base} as above. If $K_{base} \ll 1$, the solvent composition is adequate for separation.
7. If $K_{base} \geq 0.2$, add more base and retest.

A concentration of 5% ligand (dodecylamine) in the stationary phase was used for the separation of the monosulfonted components of D&C Yellow No. 10, while a higher concentration (up to 20%) of ligand was required for the separation of the di- and trisulfonated components from Quinoline Yellow (including Yellow No. 203).

Ion-pairing mode separations (**Figure 8**) The following steps are recommended for the selection of an appropriate two-phase solvent system for the separation of sulfonated dyes by affinity-ligand pH-zone-refining CCC in the ion-pairing mode:

1. Follow steps 1 and 2 as described above for the ion exchange mode separations.
2. Add a small amount of ion-pairing reagent (tetrabutylammonium hydroxide, TBAOH, 40% weight solution in water, 0.4 mmol) to give a concentration of approximately 100 mmol L^{-1} and a pH of 12.8. Equilibrate the mixture by agitation. Measure the partition coefficient K_{base}, as described above.
3. If $K_{base} \leq 5$, add more ion-pairing reagent and retest.
4. If $K_{base} \gg 1$, add an organic acid, TFA (eluter), to give an acid concentration of approximately 80 mmol L^{-1} and a pH of 1.6. Re-equilibrate the mixture by agitation. Measure the partition coefficient K as above. If $K_{acid} \ll 1$, the solvent composition is adequate for separation.

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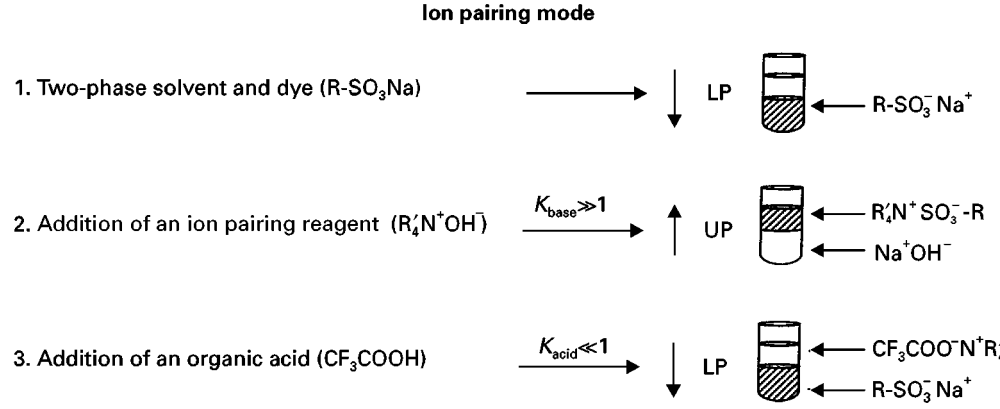

Figure 8 Conditions required for the separation of sulfonated dyes by affinity-ligand pH-zone-refining CCC in the ion-pairing mode.

5. If K_{acid} is not small enough (greater than 0.2), add more TFA and retest.

Separation Procedure

Standard pH-zone-refining CCC A standard separation is initiated by completely filling the column with the acidified (or basified) organic stationary phase using the liquid chromatography pump (Figure 2). Then the sample solution is loaded into the column through the sample injection valve by syringe or with pressurized nitrogen (60–80 psi). When dyes that contain a carboxylic group are separated, the sample solution is prepared as follows: the sample is dissolved in the organic stationary phase containing the retainer (TFA) and a smaller amount of aqueous lower phase (free of eluter). The sample solution should have a low pH, thus ensuring that the target dye is distributed into the upper (stationary) phase of the sample solution. It is desirable that the sample be completely dissolved in the sample solution. If the sample does not dissolve completely in the sample solution, it can be loaded into the column if it can be homogenized into a fine suspension by sonication for a short time. Ideally, the volume of the sample solution should not exceed 100–140 mL for a preparative separation column of 325 mL capacity. After the sample solution is loaded into the column, the mobile phase containing the eluter (basified aqueous phase) is then pumped (usually at 3 mL min^{-1}) into the column while the column is rotated (800–1000 rpm). The effluent is passed through a UV-visible detector flow cell that is further connected to a pH flow cell (for continuous pH monitoring; alternatively, the pH of each collected fraction is manually recorded with a pH meter) and fractions – usually 3 mL per test tube – are collected with a fraction collector.

Affinity-ligand pH-zone-refining CCC *Ion exchange mode* A separation is initiated by completely filling the column with ligand-free stationary phase (upper organic phase) by using the liquid chromatography pump. Approximately 100 mL of ligand-containing stationary phase is pumped into the column, thereby displacing part of the column contents. Then the sample solution is loaded into the column through the sample injection valve by syringe or with pressurized nitrogen (60–80 psi). The sample solution is prepared as follows: the sample is dissolved in lower aqueous phase (e.g. 60 mL for 5 g of Quinoline Yellow) and mixed with stationary phase (60 mL) containing the ligand. The sulfonated dyes are brought into the upper phase of the sample solution by the cautious addition of H_2SO_4 (95–98%, 2.2 mL), at which point the pH of the sample solution is approximately 0.8.

After the sample solution is loaded into the column, the aqueous mobile phase containing the basic eluter is pumped through the rotating column as described above for performing standard pH-zone-refining CCC. If carryover of the stationary phase occurs, the elution is stopped (after approximately 60 mL of stationary phase has been eluted) while the rotation of the column is continued. After 3–4 h of rotation, the elution of the mobile phase is restarted, and usually no more carryover of the stationary phase is observed.

Ion-pairing mode A separation is initiated by completely filling the column with ligand-free stationary phase (upper organic phase) by using the liquid chromatography pump. The sample solution is then loaded into the column through the sample injection valve by syringe or with pressurized nitrogen (60–80 psi). The sample solution is prepared as follows: the sample is dissolved in lower aqueous phase.

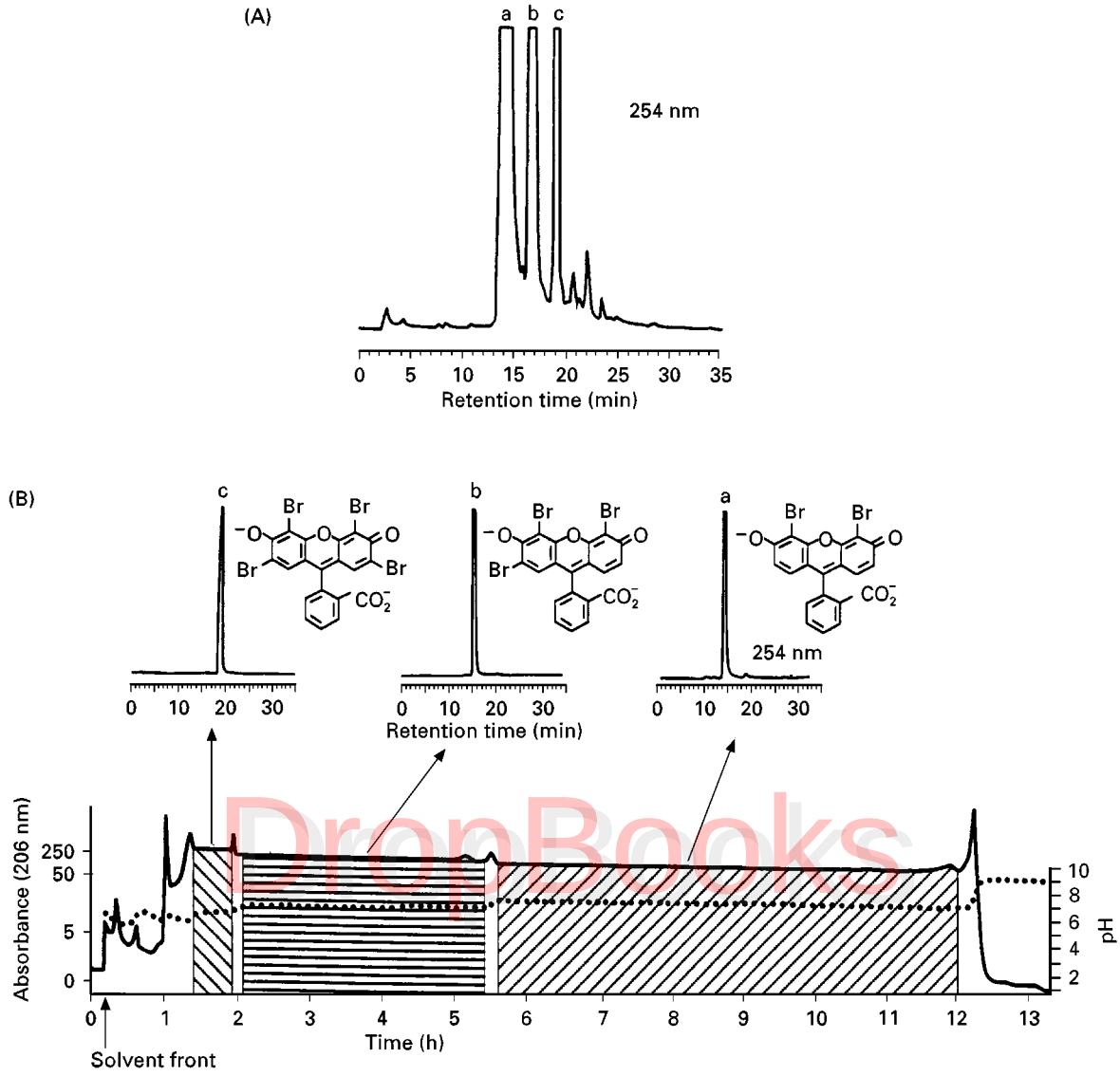

Figure 9 Separation by standard pH-zone-refining CCC of components from the colour additive D&C Orange No. 5 (CI 45370 : 1). (A) HPLC analysis of the original sample. (B) pH-zone-refining CCC of the separation and the HPLC chromatograms of the separated components. Continuous line, absorbance (206 nm); dotted line, pH. Experimental conditions: solvent system: diethyl ether–acetonitrile–0.01 mol L^{-1} aqueous ammonium acetate adjusted to pH 9 with aqueous NH_3 (4 : 1 : 5 by volume). The aqueous phase was used as mobile phase. Sample: 5 g D&C Orange No. 5 suspended in 80 mL solvent system (40 mL each of the upper and lower phases). TFA 200 μl was added to the sample solution as a retainer. Flow rate: 3 mL min^{-1} in the head-to-tail elution mode. Speed of revolution: 800 rpm. Detection: 206 nm. (Weisz *et al.*, 1994 with modifications.)

To this solution an equal volume of upper phase that contains approximately 100 mmol L^{-1} of the ion-pairing reagent (TBAOH) is added. The pH of the sample solution is approximately 12.8, and the dye partitions mostly into the upper phase. After the sample solution is loaded into the column, the mobile phase, consisting of the aqueous lower phase and 80 mmol L^{-1} TFA as an acid eluter, is pumped through the rotating column as described above for standard pH-zone-refining CCC. The pH of the mobile phase is approximately 1.6. If carryover of the stationary phase occurs, follow the directions given above for the ion exchange mode separation.

Applications

Figures 9–13 show successful applications of pH-zone-refining CCC to the preparative separation of components of fluorescein and sulfonated dyes.

Standard pH-zone-refining CCC separations Figure 9 shows the separation of the three brominated homologues contained in the colour additive D&C

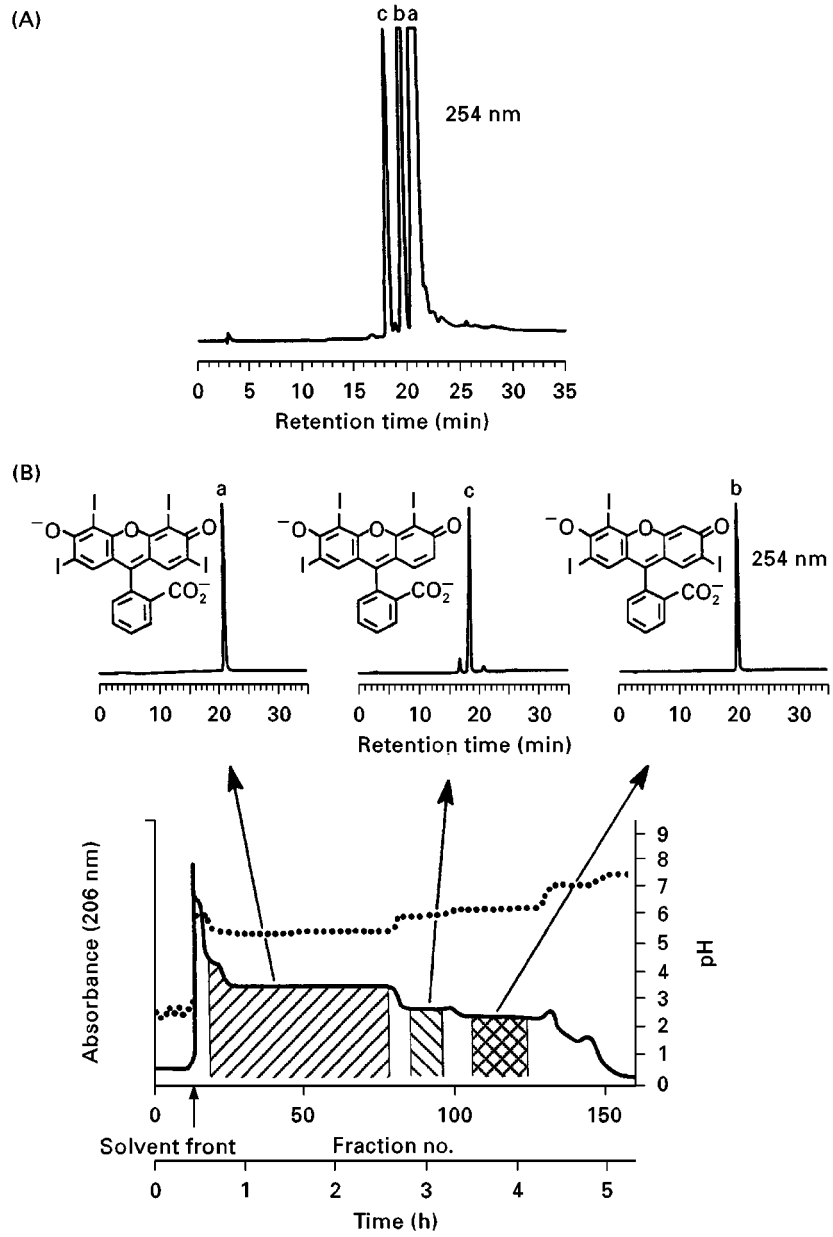

Figure 10 Separation by standard pH-zone-refining CCC of components from the colour additive FD&C Red No. 3 (erythrosine, CI 45430). (A) HPLC analysis of the original sample. (B) pH-zone-refining CCC of the separation and HPLC analyses of the separated components. Continuous line, absorbance (206 nm); dotted line, pH. Experimental conditions: solvent system: diethyl ether–acetonitrile–0.01 mol L^{-1} aqueous ammonium acetate (4 : 1 : 5 by volume). The aqueous phase, adjusted to pH 7.53 with aqueous NH_3, was used as mobile phase. The organic phase (500 mL), to which was added TFA (400 μL) as a retainer, was used as stationary phase. Sample: 3 g FD&C Red No. 3 suspended in 40 mL solvent system (20 mL of the lower phase and 20 mL of the unacidified upper phase). Flow rate: 3 mL min^{-1} in the head-to-tail elution mode. Speed of revolution: 800 rpm. Detection: 206 nm. (Weisz, 1996 with modifications.)

Orange No. 5 (CI 45370:1) by standard pH-zone-refining CCC. The HPLC chromatogram of the original sample is shown in Figure 9A. The pH-zone-refining CCC chromatogram of a suspension containing 5 g of this mixture is shown in Figure 9B. The three broad absorbance plateaux (solid line) correspond to the three pH plateaux. Each plateau represents elution of a pure component of the mixture, as illustrated by the associated HPLC chromatograms of aliquots of the combined fractions from the three hatched regions. The recoveries of the three dye components were good (a: 82%; b: 90.3%; c: 77%).

Figure 10 shows the use of standard pH-zone-refining CCC for the separation of iodinated homologues

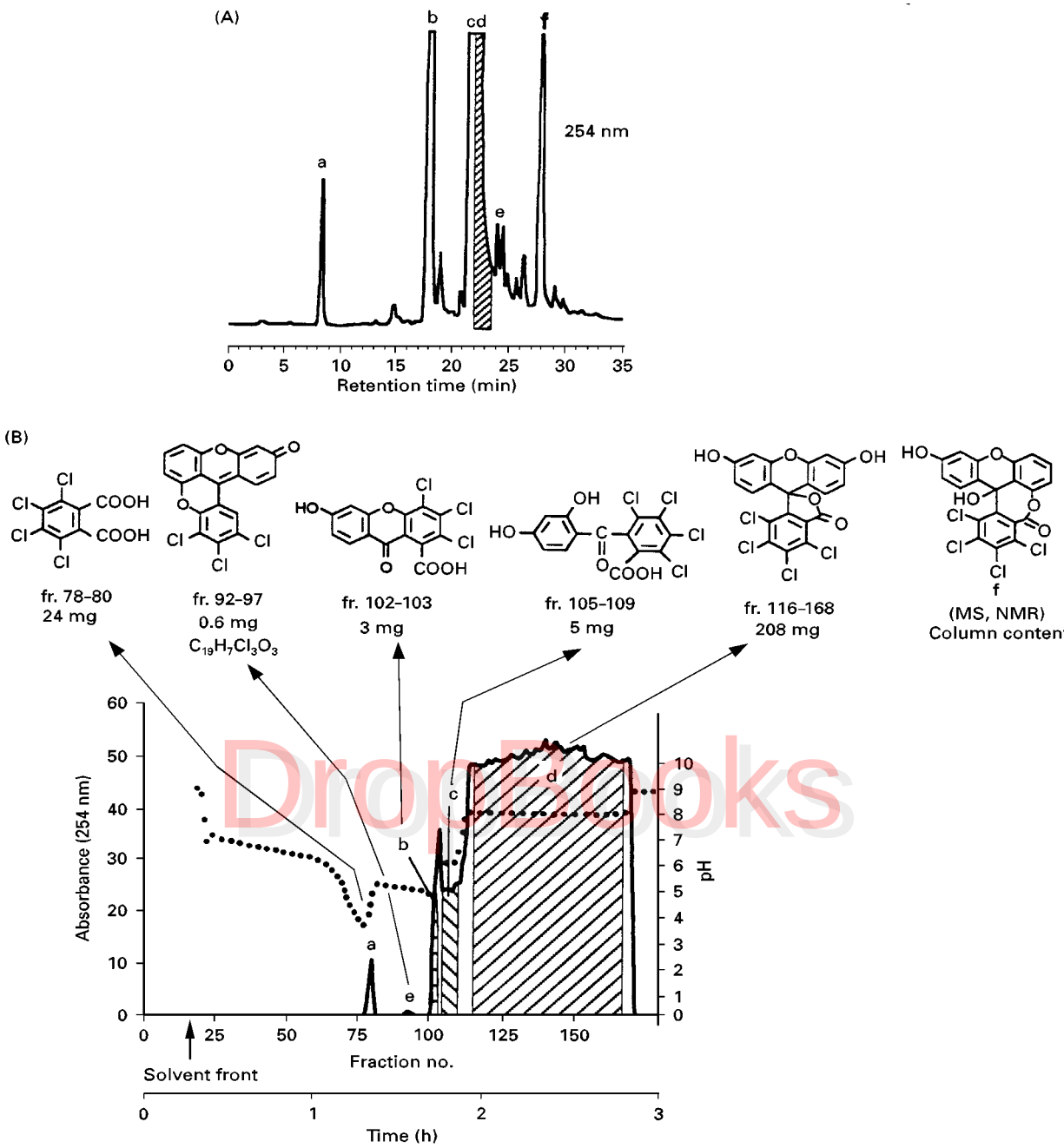

Figure 11 Separation by standard pH-zone-refining CCC of components from a sample of tetrachlorofluorescein (TCF). (A) HPLC analysis of the original mixture. (B) pH-zone-refining CCC of the separation and the assigned structures of the isolated components. Continuous line, absorbance (206 nm); dotted line, pH. Experimental conditions: solvent system: diethyl ether–acetonitrile–0.01 mol L^{-1} aqueous ammonium acetate (4 : 1 : 5 by volume). The aqueous phase adjusted to pH 9.06 with aqueous NH_3 (0.1%, c. 14 mmol L^{-1}) was used as mobile phase. The organic phase was used as stationary phase. Sample: 350 mg TCF suspended in 5.5 mL of each of upper and lower phases. TFA (200 μL) was added as a retainer in the sample solution. Flow rate: 3 mL min^{-1} in the head-to-tail elution mode. Speed of revolution: 800 rpm. Detection: 254 nm. Retention of the stationary phase: 74.8%. (Weisz *et al.*, 1995 with modifications.)

and positional isomers from a 3 g sample of the colour additive FD&C Red No. 3 (erythrosine, CI 45430). All three components were well-separated with small mixing zones between them.

Figure 11 illustrates the separation of the main component and five contaminants from a sample of 350 mg of commercial tetrachlorofluorescein (TCF). TCF is an important intermediate in the preparation

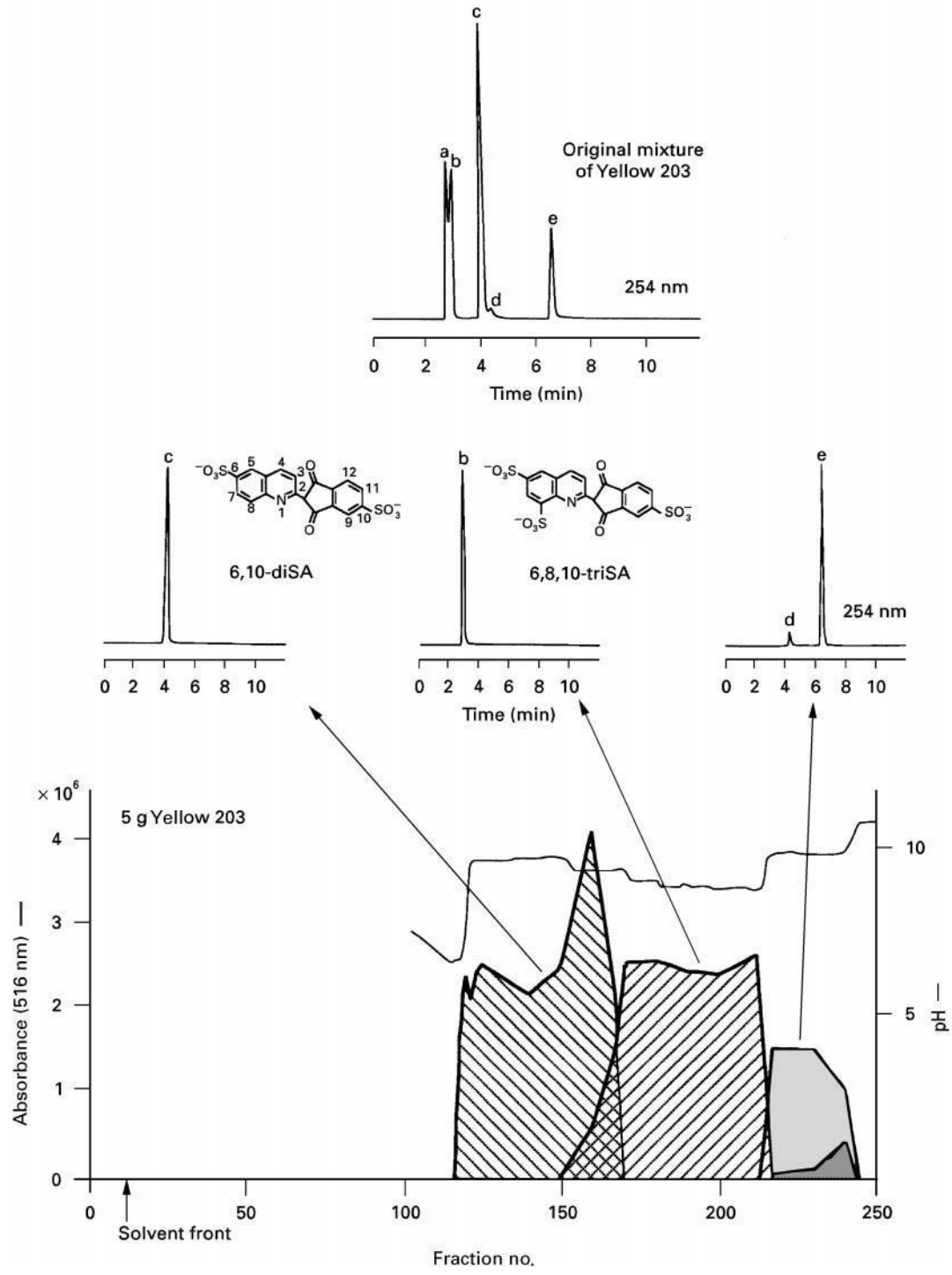

Figure 12 Separation by affinity-ligand pH-zone-refining CCC in the ion exchange mode of components from a sample of the Japanese colour additive Yellow No. 203 (Quinoline Yellow, CI 47005). Top: HPLC analysis of the original mixture. Bottom: reconstructed pH-zone-refining CCC elution profile (a constant amount from each CCC collected fraction was analysed by HPLC and the area of the peak obtained at 516 nm was plotted). Experimental conditions: solvent system: iso-amyl alcohol–methyl-*tert*-butyl ether–acetonitrile–water (3 : 5 : 1 : 7 by volume). Stationary phase: 100 mL upper phase, to which was added 20% dodecylamine as a retainer (pH 11.7) and 220 mL of ligand-free upper phase (see text). Mobile phase: lower phase, to which was added aqueous NH_3 (163 mmol L^{-1}, pH 11.7). Sample solution: 5 g of Yellow No. 203 dissolved in 60 mL each of lower phase and ligand-containing upper phase. To this mixture was added conc. H_2SO_4 (40 mmol). The pH of the sample solution (light line, top) became 0.8 and the dye partitioned mostly in the upper phase. Flow rate: 3 mL min^{-1} (see text). Speed of revolution: 800 rpm. Detection: 516 nm (bold line). Retention of the stationary phase: 36.1%. Time of the separation: equilibration 12 h; actual separation time: 5 h. For more details, see text. Reproduced from Weisz *et al.* (1995) with permission from the American Chemical Society.

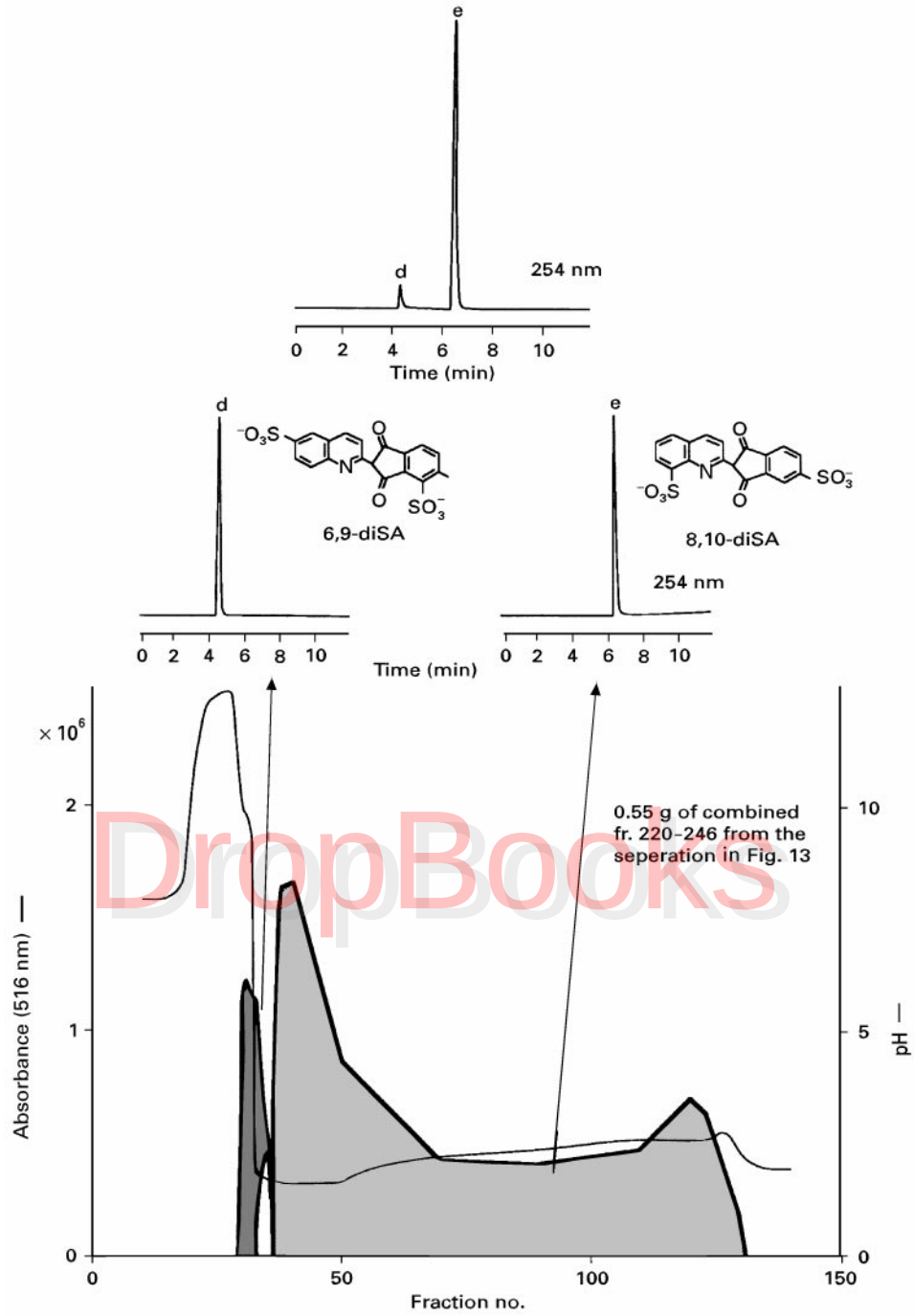

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Figure 13 Separation by affinity-ligand pH-zone-refining CCC in the ion-pairing mode of the two components of Yellow No. 203 that elute together in the separation shown in Figure 12. Top: HPLC analysis of the combined fractions 220–246 from the separation in Figure 12. Bottom: reconstructed pH-zone-refining CCC of the separation and HPLC analyses of the separated components. Experimental conditions: solvent system: iso-amyl alcohol–methyl-*tert*-butyl ether–acetonitrile–water (3 : 4 : 1 : 7 by volume). Stationary phase: upper phase. Mobile phase: lower phase to which was added TFA (80.7 mmol L^{-1}, pH 1.6). Sample solution: 0.55 g of residue from combined fractions 220–246 from the separation in Figure 12 dissolved in 30 mL of solvent system (15 mL each of lower and upper phases), to which was added tetrabutylammonium hydroxide (100 mmol L^{-1}). The pH of the sample solution (light line, top) became 12.8 and the dye partitioned mostly in the upper phase. Flow rate: 3 mL min^{-1}. Speed of revolution: 825 rpm. Detection: 516 nm (bold line). Retention of the stationary phase: 75%. Time of the separation: 2.5 h.

of higher halogenated dyes (e.g. Phloxine B, Rose Bengal), and its contaminants can be carried over during the manufacturing process. Two of the isolated contaminants (e and f) had not been reported previously.

Affinity-ligand pH-zone-refining CCC separations

Ion exchange mode **Figure 12** shows the separation of a 5 g portion of the Japanese colour additive Yellow No. 203 (Quinoline Yellow, CI 47005) with the ion exchange reagent dodecylamine as the retainer in the stationary phase at a concentration of 20%. To retain the stationary phase in the column, at the point in the separation when the column contains approximately 50% of the mobile and 50% of the stationary phase, reduce the flow rate of the mobile phase to one-fifth of the original flow rate (i.e. 0.6 mL min^{-1} instead of 3 mL min^{-1}) for several hours, and then the flow rate is increased to its original value. This procedure results in a satisfactory retention of the stationary phase of 36.1% (measured at the end of the separation). A good separation was obtained for two major components, c and b. The first few fractions containing c were contaminated with component a (not shown in **Figure 12**). Two other components (d and e) eluted together, as shown by the associated HPLC chromatograms in **Figure 12**.

Ion-pairing mode By using an ion-pairing reagent (TBAOH) as the retainer in the sample solution containing the two components of Yellow No. 203 (0.55 g) that eluted together in **Figure 12**, d and e were well-separated, as shown in **Figure 13**. Compound d is a newly identified disulfonated positional isomer of Quinoline Yellow. The results presented in **Figures 12** and **13** for the separation of the components of Yellow No. 203 demonstrate that the ion exchange and ion-pairing modes can complement each other.

Conclusion

HSCCC has been used to accomplish the difficult task of separating and/or purifying multi-gram amounts of dyes containing either sulfonic or carboxylic acid groups. No other preparative-scale chromatographic separation of multi-gram quantities of these dyes has been previously reported. It is envisioned that HSCCC could be scaled up to separate kilogram quantities of dyes through modifications of the instrumentation. The availability of large quantities of highly purified dyes will have many benefits, such as enabling standardization of biological stains and lowering the cost of the ultra-pure laser-quality dyes.

See also: **II / Chromatography:** Countercurrent Chromatography and High-Speed Countercurrent Chromatography Instumentation. **III / Dyes:** High-Speed Countercurrent Chromatography.

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Liquid Chromatography

W. Nowik, Laboratoire de Recherche des Monuments Historiques, Champs-sur-Marne, France

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Introduction

Natural dyes are organic matter made up of coloured compounds originating from natural living sources such as plants and animals. These compounds can be found either directly in the extracts or become coloured by hydrolysis, oxidation, condensation, etc., from extracted colourless precursors. Some of them change colour following such reactions or by complexation with certain metallic cations.

These dyestuffs are generally employed for dyeing fibres and fabrics (usually protein and cellulosic), staining several organic and mineral materials (histological preparations, wood, hair, feathers, Easter egg shells), producing organic pigments (painting, printing) and colouring of food, beverages, cosmetics and pharmaceutical products. They are used in analytical chemistry as well as acid–base indicators or complexation agents. A large number of naturally occurring coloured substances are chemically labile. This excludes their direct use principally because of poor light-fastness. Some dyestuffs, in addition to their colouring qualities, have therapeutic properties like regulation of metabolism, anti-inflammatory treatment and anti-cancer prevention among others.

Used frequently around the world in traditional societies, starting probably from the Stone Age, natural dyes were rapidly replaced by synthetic dyes from the second half of the nineteenth century onwards. Nowadays, natural dyes have a growing importance in our lives since almost all of them are hypoallergenic and nontoxic for humans, which is a significant advantage over many synthetic dyes.

Chemical classification of dyestuffs follows the general arrangement of organic compounds and depends on the generic, basic structure. There are over 20 family structures which many hundreds of compounds belong to. Examples of the most frequently encountered structures are shown in **Table 1**.

The most important application of dyes, from a historical point of view, is textile dyeing. For this reason, the most common classification of dyestuffs is determined by their dyeing properties and dyeing technology. According to their dyeing properties, natural dyes belong to four principal groups: direct dyes (e.g. curcumin, a diaryloylmethane from *Curcuma longa*), mordant dyes (e.g. alizarin and other anthraquinones from various madders), reactive dyes (e.g. depsides and depsidones from lichens) and vat dyes (e.g. indigotine from indigo, woad, dyer's knotweed and others).

Membership to one of the dye families is determined by the type of 'connection' between colorant and support. However, many dyestuffs may show more than one dyeing mechanism, simultaneously or according to applied dyeing conditions. The type of connection determines the method of liberation of dyestuff prior to analysis. Identification of type and origin of dyestuffs requires an understanding of composition.

Extraction

Direct Raw Material Extraction

Many natural dyestuffs or their precursors (e.g. indican and isatan B for indigotine) are water-soluble. Their extraction from raw plant or animal material can be achieved by water, sometimes heated to facilitate penetration into the cells. This process, along with possible cell destruction, can also be accelerated by the use of an ultrasound bath. In several cases, the addition of water-miscible solvents (e.g. methanol, ethanol or acetonitrile) improves the recovery of relatively hydrophobic compounds, such as curcumin, certain antraquinons, etc. Moderately polar solvents

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